Mortality in gerbils with repetitive ischemia: CGS-19755/hypothermia therapy

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Abstract
Repetitive ischemia causes more severe damage than a single insult of comparable duration. Gerbils were followed for 1 month postrepetitive ischemia and 100% mortality was demonstrated in the unprotected ischemia group by 12 days postischemia. Significant protection against mortality due to repetitive ischemia was offered by both CGS-19755 and combination CGS-19755-hypothermia treatments. Current practices of sacrificing repetitive ischemia subjects shortly postischemia may lead to an underestimation of the effects of ischemia and/or an overestimation of the protective effects of experimental treatments.

Key words: CGS-19755; Hypothermia; Repetitive ischemia; Delayed neuronal death; Mortality

There is currently much interest in understanding the mechanisms of neuronal damage during and after cerebral ischemia in models of global ischemia [3]. Whereas the damage in the CA4 region of the hippocampus begins almost immediately and is well advanced within 24 h, damage in the CA1 region of the hippocampus begins 2–3 days after the restoration of cerebral blood flow. This new damage after blood flow has been re-established is known as ‘delayed neuronal damage’ and has been observed in gerbils [4,10] and humans [16]. There is an increase in damage if brief episodes of ischemia are repeated during the reperfusion period [12,14]. Furthermore, the effect of such repetitive cerebral ischemia is cumulative and exceeds the damage from a single insult of similar duration [11,9,15]. Recently, it was demonstrated that the administration of cis-4-(phosphonomethyl)-2-piperidine carboxylic acid (CGS-19755), a potent competitive NMDA receptor blocker in combination with hypothermia to repetitive ischemic gerbils significantly reduced brain damage in comparison to controls [21]. In this study, an examination of the neuroprotective properties of these two treatments over the longterm was carried out on gerbils postrepetitive ischemia. Most previous investigations employing the repetitive ischemia model have sacrificed their animals within a week of the ischemia procedure [1,2,6–9,11,14,18–23].

Fifty-two male Mongolian gerbils (60–80 g: Tumblebrook Farms, West Brookfield, MA) were used for this study. After acclimatization to a 12-h day-night cycle with free access to food and water, the animals underwent surgery. The experimental design included five groups: a sham-saline injection group (n = 8), an ischemia-saline injection group (n = 11), an ischemia-hypothermia-treated and saline-injected group (n = 10), an ischemia-CGS-19755-injected group (n = 11), and finally an ischemia-hypothermia-treated and CGS-19755-injected group (n = 10).

Animals were subjected to repetitive forebrain ischemia using a procedure similar to that described by Shuaib et al. [18–21]. Subjects in which hypothermia was induced had their whole body temperatures decreased to 34–35°C during the two 1-h reperfusion periods and for...
0.5 h after the third occlusion. This procedure is described in detail elsewhere [20]. CGS-19755 in isotonic saline solution was administered by the i.p. route at a dose of 30 mg/kg immediately after each of the three ischemic insults, 6 h after the first insult and, finally, 12 h after the first insult, for a total of five injections. Non-drug-treated subjects received an experimental control injection of saline in the same manner.

At 1 month postischemia, each surviving animal was anaesthetized with an overdose of pentobarbitol and perfused through the left heart. The brains were removed from the skulls, sliced coronally, stained and rated blindly with light microscopy in a number of forebrain areas. This perfusion, staining and rating procedure was identical with that previously described [19].

Due to 100% mortality in the ischemia-saline group, it proved impossible to assess the degree of brain damage in that group. Statistical analyses (Kruskal–Wallis test) revealed that the CGS-19755 group, the hypothermia group and the CGS-19755-hypothermia group each had significant damage in six of seven brain areas tested as compared with the sham-saline group: cortex (P > 0.06); CA1, CA4, striatum, thalamus, medial geniculate nucleus, all (P < 0.01); and substantia nigra (P < 0.05). Individual Mann–Whitney comparisons revealed that the only difference between the three experimental groups was that the drug-hypothermia group was less damaged in the CA4 cell field (P < 0.005) than the drug group.

Analysis of the overall mortality effects among the five groups with the Kruskal–Wallis H test revealed an overall significant effect (P < 0.01). Individual comparisons (Mann–Whitney U test) revealed that the ischemia-saline group (100% mortality) demonstrated a significantly higher overall mortality rate than the sham (P < 0.002), drug (P < 0.01) and hypothermia-drug group (P < 0.002). The hypothermia group at 55% mortality, was not significantly different from any other group. The lack of a significant difference in mortality between the drug and drug-hypothermia groups suggests that the drug treatment was critical in the enhanced survivability of these two groups over the longterm (Fig. 1).

In a review of the clinical importance of the excitatory neurotransmitter glutamate, it was implicated directly in the etiology of ischemic brain damage postischemia [3]. As well abnormal calcium ion fluxes, as a consequence of short duration ischemia, may also play a central role in delayed neuronal degeneration postischemia [1].

Glutamate antagonists, such as CGS-19755, exert neuroprotective properties postischemia [21] as do (GABA)-ergic agents which inhibit neuronal excitability [18]. Abnormally high calcium accumulation immediately postrepetitive ischemia in vulnerable brain areas in the gerbil brain and subsequent neuronal death in these areas, 7 days later, have been highly correlated [11]. Recently, investigators demonstrated that the administration of calpain inhibitors (calpains are calcium-binding proteins) 2 h and 30 min prior to ischemia offers significant neurological protection in rats sacrificed at 7 days postischemia [17]. Mechanisms suggested to be responsible for the neuroprotective effects of mild hypothermia include: a slowdown in metabolism, reduced excitatory amino acid release, decreased lactate production, prevention of cerebral edema and inhibition of breakdown of adenine nucleotide [20].

The mechanisms of delayed neuronal death postrepetitive ischemia are not as well understood as the mechanisms of single insult ischemic processes. Edema of the brain tissue in the reperfusion phase of the repetitive ischemia insult as a result of hypoperfusion [23] as well as abnormalities in brain protein synthesis postrepetitive ischemia have been implicated as possible factors contributing to the greater brain damage of this model over the single insult [11,12].

The failure of hypothermia alone to protect; while drug plus hypothermia resulted in less CA4 damage than drug treatment could be explained in light of a recent study. This study suggested that hypothermia alone only delays inevitable neuronal death and that pharmacological interventions, such as that used in this study, are needed to arrest later-occurring pathophysiological events postischemia [5]. The combination-treated group was, thus, given the most potent protection in the ischemically vulnerable CA4 since early and late-occurring pathophysiological processes were both arrested.

Areas, such as the substantia nigra and striatum, were heavily damaged in this study and in others [18–21]. The characteristic worsening ataxia and inevitable 100% mortality in untreated subjects have been observed in other experiments in our laboratory. Since these regions are involved in limbic-motor integration [13], the ataxia resulting from the repetitive ischemic procedure appears to be so severe as to handicap normal feeding, drinking and body maintenance Functions like grooming.

The significantly decreased mortality rate in the ischemia-drug group and the highly significant reduction
in mortality in the combination-treated group as compared with the ischemia-saline control group is suggestive of the neuroprotective efficacy of CGS-19755 and, especially, of a combination CGS-19755 and hypothermia treatment against repetitive ischemia. These findings are consistent with a recent study by Shuaib et al. [21].

Many previous studies utilizing the repetitive ischemia procedure have done neuropathological analyses before 7 days postischemia. In light of our findings, the debilitating effects of the procedure may have been underestimated and and more importantly the protective effects of various agents may have been overestimated in such experiments. Recently, other researchers have suggested that histological assessments should only take place after prolonged survival, perhaps on the order of several weeks postischemia, because of the persistence of delayed neuronal death processes up until this time [5].

[21] Shuaib, A., Ijaz, S., Mazagri, R. and Sennihisvelan, A., CGS-19755 is neuroprotective during repetitive ischemia: this effect is significantly enhanced when combined with hypothermia, Neuroscience, in press.